Anthelmintic activity of *Cocos nucifera* L. against sheep gastrointestinal nematodes


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**1. Introduction**

Breeding of small ruminants is a widespread economic activity in northeastern Brazil. However, gastrointestinal nematodes limit the productivity, besides causing high mortality rates in herds during the rainy season (Pinheiro et al., 2000). Epidemiological investigations carried out in this region have shown that sheep and goats are infected by nematodes and that *Haemonchus contortus* is the most prevalent species (Silva et al., 2003).

In recent decades, gastrointestinal nematode control has generally been based on continuous and intensive use of synthetic anthelmintics. However, the high cost of these drugs and the development of nematode-resistant populations (Melo et al., 2003), along with the risk of contamination of the animal products (Herd, 1995) and environment (Hammond et al., 1997) have spurred the search for control alternatives. Among these is the use of medicinal plants, since they have the advantage of sustainable supply and are ecologically acceptable.

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**ABSTRACT**

The development of anthelmintic resistance has made the search for alternatives to control gastrointestinal nematodes of small ruminants imperative. Among these alternatives are several medicinal plants traditionally used as anthelmintics. This work evaluated the efficacy of *Cocos nucifera* fruit on sheep gastrointestinal parasites. The ethyl acetate extract obtained from the liquid of green coconut husk fiber (LGCHF) was submitted to *in vitro* and *in vivo* tests. The *in vitro* assay was based on egg hatching (EHT) and larval development tests (LDT) with *Haemonchus contortus*. The concentrations tested in the EHT were 0.31, 0.62, 1.25, 2.5 and 5 mg ml⁻¹, while in the LDT they were 5, 10, 20, 40 and 80 mg ml⁻¹. The *in vivo* assay was a controlled test. In this experiment, 18 sheep infected with gastrointestinal nematodes were divided into three groups (*n* = 6), with the following doses administered: G1—400 mg kg⁻¹ LGCHF ethyl acetate extract, G2—0.2 mg kg⁻¹ moxidectin (Cydectin®) and G3—3% DMSO. The worm burden was analyzed. The results of the *in vitro* and *in vivo* tests were submitted to ANOVA and analyzed by the Tukey and Kruskal–Wallis tests, respectively. The extract efficacy in the EHT and LDT, at the highest concentrations tested, was 100% on egg hatching and 99.77% on larval development. The parameters evaluated in the controlled test were not statistically different, showing that despite the significant results of the *in vitro* tests, the LGCHF ethyl acetate extract showed no activity against sheep gastrointestinal nematodes.

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Cocos nucifera L. is a monocotyledone plant belonging to the Palmae family, which is widely distributed in northeastern Brazil. The products from the fruit have been used in popular medicine in this region for the treatment of various diseases (Esquenazi et al., 2002), including gastrointestinal parasitism (Blini and Lira, 2005). The antiparasitic activity of coconut milk was tested in mice and showed efficacy against Syphacia obvelata, Aspiculuris tetraptera (Amorim and Borba, 1994) and Vampyrolepis nana (Amorim and Borba, 1995). The liquid extracted from the coconut husk fiber has antiproliferative activity against lymphocytes (Kirsberg et al., 2003). Analgesic and antioxidant activity was also found (Alviano et al., 2004). Fractions of this liquid obtained with the ethyl acetate solvent demonstrated activity against Staphylococcus aureus (Esquenazi et al., 2002) and Leishmania amazonensis (Mendonça-Filho et al., 2004). High performance liquid chromatography and mass spectrometry showed this extract is composed of catechin, epicatechin and condensed tannins (Esquenazi et al., 2002). The objective of this work was to evaluate in vitro and in vivo the anthelmintic activity of the ethyl acetate extract obtained from the liquid of green coconut husk fiber (LGCHF) against sheep gastrointestinal nematodes.

2. Materials and methods

All proceedings were approved by the Ethical Committee of Ceará State University (number: 07227499-9).

2.1. Preparation of the extract

The LGCHF was supplied by EMBRAPA/Agroindústria Tropical, located in Fortaleza, Ceará, Brazil. For this, the green coconut husk fiber, a byproduct of food processing industries, was crushed and pressed to obtain the liquid, which was filtered and stored at −20 °C until use. Three hundred liters of LGCHF were placed in a decanting funnel and submitted to three washes with ethyl acetate in 10:1 proportion. The solvent was eliminated in a rotary evaporator to produce LGCHF ethyl acetate extract. To increase the solubility in aqueous medium, the extract was diluted in 3% dimethylsulfoxide (DMSO).

2.2. Phytochemical analysis and total tannin quantification of the extract

The phytochemical tests to detect the presence of phenols, tannins, catechins, leucoanthocyanidins, flavonoids, steroids, triterpens, saponnins and alkaloids were performed following the method described by Matos (1997). Theses tests are based on visual observation of color modification or precipitate formation after addition of specific reagents. The total tannin quantification was performed by the Folin-Denis spectrophotometric method according to Pansera et al. (2003). For this test, 5 mg LGCHF ethyl acetate extract was diluted in 100 ml distilled water and 2 ml of this solution was added to 2 ml of Folin-Denis reagent. Subsequently, the mixture was vigorously shaken and left at rest for 3 min. Then, 2 ml of 8% sodium carbonate aqueous solution was added to the mixture, which was shaken again and left at rest for 2 h. Solutions ranging from 2 to 24 mg l⁻¹ tannic acid diluted in water were prepared to quantify total tannins. The absorbance was measured at 725 nm and a negative control was performed at each reading. The readings with three replicates per sample were performed in a spectrophotometer. An analytical calibration curve was plotted from the results.

2.3. In vitro anthelmintic activity of the extract: egg hatching and larval development tests on H. contortus

H. contortus eggs were recovered as described by Hubert and Kerboeuf (1992). About 10 g of feces were collected directly from an experimentally infected sheep, mixed with distilled water and filtered through sieves with 590, 149, 101 and 30 μm mesh apertures. An aliquot of egg suspension was incubated for 24 h at 37 °C to obtain first-stage larvae (L1).

The extract was diluted in distilled water solution with 3% DMSO. The anthelmintics thiabendazole and ivermectin used as positive control in the egg hatching and larval development tests were prepared in distilled water solution with 3% DMSO and distilled water, respectively.

The egg hatching test (EHT) was based on the methodology described by Coles et al. (1992). Two hundred and fifty microliters of egg suspension, containing approximately 100 fresh eggs, and 250 μl of LGCHF ethyl acetate extract at concentrations of 0.31, 0.62, 1.25, 2.5 and 5 mg ml⁻¹ were incubated for 48 h at room temperature. Then drops of Lugol were added. The eggs and L1 were counted under a microscope. This test had two controls: a negative with 3% DMSO and a positive with 0.025 mg ml⁻¹ thiabendazole. Three repetitions with five replicates for each extract concentration and for each control were performed.

For the larval development test (LDT), 1 ml of larval suspension, containing approximately 500 L1, and 1 ml of LGCHF ethyl acetate extract at concentrations of 5, 10, 20, 40 and 80 mg ml⁻¹ were incubated with 2 g of feces from a nematode-free sheep for 6 days at room temperature. Then the third-stage larvae (L3) were recovered according to Roberts and O’Sullivan (1950) and counted under a microscope. This test had two controls: a negative with 3% DMSO and a positive with 0.008 mg ml⁻¹ ivermectin. Three repetitions with five replicates for each extract concentration and for each control were performed.

2.4. Acute toxicity of the extract on mice

Swiss albino mice (n = 96) of both sexes, with average weight of 27.5 g, were kept in polypropylene boxes and fed with commercial feed (Fris-Ribeiro) and water ad libitum. The mice were randomly divided into 12 groups (n = 8): G1 to G5—received 1000, 1500, 2000, 2500 and 3000 mg kg⁻¹ LGCHF ethyl acetate extract by oral administration; G6—3% DMSO by oral administration; G7 to G11—500, 1000, 1500, 2000 and 2500 mg kg⁻¹ LGCHF ethyl acetate extract by intraperitoneal administration; G12—3% DMSO by intraperitoneal administration. After 24 h, the number of dead animals was verified.
2.5. In vivo anthelmintic activity of the extract: controlled test in sheep

Sheep (n = 18) of undefined breed, of both sexes, aged from 6 to 12 months and with average weight of 21 kg, were kept in paddocks and fed with fresh grass, mineral salt and water ad libitum. Individual fecal samples were collected to determine the level of gastrointestinal nematode infection by using a modified McMaster technique (Ueno and Gonçalves, 1998). The animals with egg counts per gram of feces (egp) less than 1000 were inoculated three times with 1500 H. contortus L3 on alternate days. The sheep with egg counts higher than 1000 were inoculated with a single dose of 1500 H. contortus L3. After 21 days, another egp was carried out and the sheep were divided into three homogeneous (mean egp of the group 6000) groups (n = 6): G1—received 400 mg kg⁻¹ LGCHF ethyl acetate extract for three consecutive days; G2—a single dose of 0.2 mg kg⁻¹ moxidectin (Cydectin®); G3—3% DMSO for 3 consecutive days. The protocol was by oral administration. Seven days after the end of treatment, the sheep were euthanized and submitted to necropsy to count the worm burden of the abomasum, small and large intestines (Gaba et al., 2006).

2.6. Statistical analysis

The efficacy of each treatment in the egg hatching test was determined by the formula: L1/(L1 + eggs) × 100, while in the larval development test the formula was: (L3 in the negative control group – L3 in the treated group)/L3 in the negative control group × 100. The results of the in vitro tests were expressed as mean efficacy percentage of egg hatching or larval development inhibition ± standard deviation. Data were analyzed by ANOVA and compared by the Tukey test (P < 0.05), the effective concentration to inhibit 50% (EC₅₀) of egg hatching and larval development, 95% confidence limits, R² and hill slope were determined using the Prism 3.0 program.

The lethal doses to kill 50% (LD₅₀) and 10% (LD₁₀) of the mice were calculated for each administration route from the acute toxicity test by SPSS 8.0 for Windows.

In the controlled test, the efficacy of each treatment was determined by the formula: (worm burden of negative control group – worm burden of treated group)/worm burden of negative control group × 100. The results of the in vivo test were expressed as mean efficacy percentage of worm burden reduction ± standard deviation. They were analyzed by ANOVA and compared by the Kruskal–Wallis test (P < 0.05) using the Prism 3.0 program.

3. Results

The yield of ethyl acetate extract from the 300 l of LGCHF, after solvent evaporation, was approximately 210 g. The phytochemical analysis revealed the presence of catechins, condensed tannins, flavonoids and steroids. The quantification of total tannins was 25.87%.

Table 1 shows the mean efficacy of LGCHF ethyl acetate extract on H. contortus egg hatching. The EC₅₀ in the egg hatching test was 2.20 mg ml⁻¹, 95% confidence limits ranged from 2.08 to 2.32 mg ml⁻¹. The R² and hill slope were 0.96 and 4.23, respectively.

Table 2 presents the mean efficacy of LGCHF ethyl acetate extract on H. contortus larval development. The EC₅₀ in the larval development test was 40.56 mg ml⁻¹, 95% confidence limits ranged from 35.46 to 46.40 mg ml⁻¹. The R² and hill slope were 0.97 and 2.37, respectively.

At all concentrations tested, LGCHF ethyl acetate extract presented no acute oral toxicity in mice. The LD₅₀ and LD₅₀ calculated after intraperitoneal administration of the extract were 650 (0.44–1038.5) mg kg⁻¹ and 1233.9 (237.68–2089.14 mg kg⁻¹), respectively.

Table 3 demonstrates the mean efficacy of LGCHF ethyl acetate extract on sheep worm burden. In the abomasum and small intestine only H. contortus and Trichostrongylus colubriformis were found, respectively. In the large intestine, Oesophagostomum columbianum and Trichuris ovis were present. However, due to the small number of specimens, the worm burden of this organ was not statistically analyzed. The extract’s effect did not differ significantly from that of 3% DMSO (P > 0.05). The effect on H. contortus also was not statistically different between positive and negative control groups (P > 0.05). However, moxidectin showed anthelmintic efficacy on T. colubriformis and total worm burden (P < 0.05).

4. Discussion

This work was carried out in order to find a phytotherapeutic to help control gastrointestinal nematodes in small ruminants and to provide an alternative use of a food processing byproduct. Moreover, the use of LGCHF is important from an economic, social and environmental perspective.
standpoint, because about 80–85% of the gross weight of green coconut is typically thrown out as garbage (Carrijo et al., 2002).

The LGCHF ethyl acetate extract showed ovicidal efficacy on *H. contortus* higher than other plant extracts. 50 mg ml⁻¹ of the ethyl acetate extract of *Spigelia anthelma* inhibited 100% egg hatching (Assis et al., 2003). The aqueous extract of the *Annona senegalenis* leaves inhibited 11.5% egg hatching at a concentration of 7.1 mg ml⁻¹ (Alawa et al., 2003). Maciel et al. (2006) reported 16.92% efficacy on egg hatch inhibition using 50 mg ml⁻¹ of hexane extract from *Melia azedarach* leaves. 50 mg ml⁻¹ of ethyl acetate extract from *Azadirachta indica* inhibited 51.31% of egg hatching (Costa et al., 2008). However, the larvicidal effectiveness of LGCHF ethyl acetate extract was lower than that of other plant extracts.

Although the LGCHF ethyl acetate extract showed 100% efficiency on egg hatching and 99.77% on larval development of *H. contortus*, the concentrations used to obtain these results were high, 5 and 80 mg ml⁻¹, respectively, compared with thiabendazole (0.025 mg ml⁻¹) and ivermectin (0.008 mg ml⁻¹) used as positive control. This fact can be explained by the presence of small concentrations of the active ingredient in the plant extracts (Rates, 2001), among these the compound with ovicidal and larvicidal activity, unlike synthetic anthelmintics, where the chemical compounds are isolated in pure form.

The phytochemical analysis of LGCHF ethyl acetate extract showed the presence of different chemical compounds, among them condensed tannins. This finding is consistent with the results of Esquenazi et al. (2002) in an experiment conducted to verify the antibacterial and antiviral activity of the ethyl acetate extract obtained from coconut husk fiber. Condensed tannins are polyphenolic compounds derived from plants’ secondary metabolism. Several experiments have demonstrated their anthelmintic activity (Molan et al., 2000; Athanasiadou et al., 2001; Niezen et al., 2002; Paolini et al., 2003). Two hypotheses put forward to explain the antiparasitic action of these compounds. First, the direct hypothesis, that is the ability of condensed tannins to interact with proteins of the cuticle, oral cavity, esophagus, cloaca and vulva of nematodes, changing their chemical and physical properties. Second, the indirect hypothesis, that is the capacity of condensed tannins to bind of dietary proteins and protect them from rumen degradation increasing protein flow to, and amino acid absorption by, the small intestine improving host immune response against worms (Hoste et al., 2006). However the role of these metabolites for immunity in sheep against nematodes needs to be better established. Although the high percentage of total tannins could be responsible for the *in vitro* anthelmintic effect observed, the activity of catechins, flavonoids and steroids should not be discarded. In addition, the synergistic action of the constituents present in the extract should also be considered (Rates, 2001).

After obtaining promising results in *in vitro* tests, a next step to assess the anthelmintic activity of medicinal plants is toxicology studies in laboratory animals, which will enable defining the dose to be tested in producing animals. Orally LGCHF ethyl acetate extract did not cause acute toxicity. This may be justified by poor absorption of the extract, detoxification by the liver (Loomis and Hayes, 1996) or degradation by the digestive enzymes of the stomach and gut (Spinosa et al., 2002) during oral administration, while with intraperitoneal administration systemic absorption occurs and the toxic effects are stronger and appear earlier (Loomis and Hayes, 1996). The low oral toxicity of the extract obtained from coconut husk fiber was also proven by Alviano et al. (2004).

The traditional use of *C. nucifera* against gastrointestinal parasites, the presence of condensed tannins in LGCHF ethyl acetate extract, the high levels of total tannins, the promising *in vitro* results and the low acute toxicity in laboratory animals justifies the assessment of *in vivo* anthelmintic activity of the extract. The controlled test is the most reliable method for evaluation of anthelmintic effectiveness in ruminants, with a recommendation for dose confirmation or titration (Wood et al., 1995). Extracts from *Myrsine africana* and *Rapanea melanophloeos*, traditionally used in Kenya to reduce the parasitism of small ruminants, did not decrease the epg of sheep experimentally infected with *H. contortus* (Githiori et al., 2002). The essential oil of *Chenopodium ambrosioides*, used for many years to control parasites in dogs, cats and humans, did not reduce the epg and worm burden of goats naturally and experimentally infected with *H. contortus* (Ketzis et al., 2002). The leaves of *Azadirachta indica*, known for their various medical applications, showed no anthelmintic effect in sheep naturally infected with gastrointestinal nematodes (Costa et al., 2006).

Different hypotheses can explain the lack of *in vivo* anthelmintic activity of LGCHF ethyl acetate extract: biotransformation of active substances of the extract, the extract dose or duration of treatment (Hennessy, 1997). Therefore, in accordance with the protocol used in

Table 3
Mean efficacy percentage ± S.D. of ethyl acetate extract obtained from the liquid of green coconut husk fiber (LGCHF) and mean adult nematodes ± S.D. recuperated from sheep treated.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>H. contortus</th>
<th>Trichostrongylus colubriformis</th>
<th>Worm burden</th>
</tr>
</thead>
<tbody>
<tr>
<td>400 mg kg⁻¹ ethyl acetate extract</td>
<td>Adult ± S.D.</td>
<td>261.5 ± 121.5⁴</td>
<td>271.5 ± 136.0⁴</td>
</tr>
<tr>
<td></td>
<td>Efficacy ± S.D.</td>
<td>4.77 ± 11.6⁹</td>
<td>18.69 ± 30.2⁸</td>
</tr>
<tr>
<td>0.2 mg kg⁻¹ moxidectina</td>
<td>Adult ± S.D.</td>
<td>107.0 ± 67.3⁹</td>
<td>17.83 ± 22.2⁷</td>
</tr>
<tr>
<td></td>
<td>Efficacy ± S.D.</td>
<td>28.77 ± 40.2⁶</td>
<td>93.44 ± 8.1⁸</td>
</tr>
<tr>
<td>3% dimethylsulfoxide</td>
<td>Adult ± S.D.</td>
<td>192.0 ± 147.3⁴</td>
<td>33.5 ± 222.9⁴</td>
</tr>
<tr>
<td></td>
<td>Efficacy ± S.D.</td>
<td>24.41 ± 37.8⁴</td>
<td>23.04 ± 25.5⁵</td>
</tr>
</tbody>
</table>

Capital letters compare mean adult nematodes and small letters mean efficacy. Different letters indicate significantly different values (*P* < 0.05).

L.M.B. Oliveira et al. / Veterinary Parasitology 159 (2009) 55–59
this experiment, the ethyl acetate extract obtained from the liquid of green coconut husk fiber presented no anthelmintic activity against sheep gastrointestinal nematodes.

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